AD_____

Award Number: DAMD17-00-1-0667

TITLE: TIMP-1 Regulation of ECM and Apoptosis

PRINCIPAL INVESTIGATOR: Hyeong-Reh C. Kim, Ph.D.

CONTRACTING ORGANIZATION: Wayne State University
Detroit, Michigan 48202

REPORT DATE: January 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 2002	Final (1 Oct 00 - 1 Dec 01)

4. TITLE AND SUBTITLE
TIMP-1 Regulation of ECM and Apoptosis

5. FUNDING NUMBERS
DAMD17-00-1-0667

6. AUTHOR(S)
Hyeong-Reh C. Kim, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Wayne State University
Detroit, Michigan 48202

E-Mail: hrckim@med.wayne.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

The importance of apoptosis in normal development and pathogenesis has been well recognized, and explosive progress towards dissecting its commitment step has been made during the past decade. Mitochondria, Apaf-1, caspase, and bcl-2 family members play central roles in the commitment step. However, it is still unclear how upstream cell survival pathways regulate apoptosis. It is also unknown whether the bcl-2 family members have any effect on the upstream survival pathways. Our studies demonstrate that the anti-apoptotic gene product bcl-2 greatly induces expression of the tissue inhibitor of metalloproteinase-1 (TIMP-1) in human breast epithelial cells. Surprisingly, we found that TIMP-1, like bcl-2, is a potent inhibitor of apoptosis induced by a variety of stimuli. Functional studies indicate that TIMP-1 inhibits a classical apoptotic pathway mediated by caspases, and that focal adhesion kinase (FAK)/PI 3-kinase and mitogen activated protein kinase (MAPK) are critical for TIMP-1-mediated cell survival. We show specific association of TIMP-1 with the cell surface. Consistently, a 150-kDa surface protein was identified in MCF10A cells that specifically binds TIMP-1. Taken together, we hypothesize that TIMP-1 binding on the cell surface induces a cell survival pathway that regulates the common apoptosis commitment step. Our studies address a new paradigm in the regulation of apoptosis by an extracellular molecule TIMP-1, and also greatly enhance our understanding of TIMP-1's pleiotropic activity in many physiological and pathological processes. This information may also be useful in designing more rational therapeutic interventions aimed at modulating the anti-apoptotic activity of TIMP-1.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
11

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

(4) Introduction

Previous studies in our laboratory examined the role of bcl-2 in apoptosis using breast epithelial cells genetically engineered to overexpress bcl-2 (1). Since ECM is critical for apoptosis regulation, particularly in breast epithelium, we hypothesized that the death-suppressing activity of bcl-2 is partly mediated by a complex regulation of matrix-degrading enzymes and/or their inhibitors, the TIMPs. We found that bcl-2 overexpression is associated with enhanced levels of TIMP-1 expression suggesting a role for TIMP-1 in apoptosis. Indeed, apoptosis studies with breast epithelial cells show that administration of recombinant TIMP-1 or endogenous overexpression of TIMP-1 protects against a variety of apoptotic stimuli including anoikis and free radicals. Interestingly, the death-suppressing activity of TIMP-1 is independent of the level of bcl-2 expression (2).

These results raise important basic questions regarding the biological role of TIMP-1 in breast cancer progression. For example, in breast cancer, TIMP-1 expression has been associated with poor prognosis; an unexpected finding considering that TIMP-1 is a known matrix metalloproteinase (MMP) inhibitor (3-5). Our study provides new clues about TIMP-1 function in breast neoplasia, a novel regulator of apoptosis. Therefore, it is important to understand the molecular mechanisms by which TIMP-1 exerts "oncogenic" activity in breast epithelium through apoptosis inhibition. During the funding period, we further investigated the role of TIMP-1 in breast epithelial cell apoptosis. We examined what survival signaling pathways are activated by TIMP-1, and also tested whether exogenous TIMP-1 binds on the cell surface to activate cell survival pathways.

(5) Body of Report

Methods

DEVDase activity

Cells were lysed in CEB (20 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT) containing 0.03% Nonidet. Lysates were centrifuged at 15,000 g for 10 min, and 50 µl cytosol fraction was incubated for 60 min at 37 ^{0}C in a total volume of 200 µl caspase buffer [10 mM HEPES (pH 7.5), 50 mM NaCl, and 2.5 mM DTT] containing 25 µM acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl coumarin (Ac-DEVD-AMC, Bachem, PA). Using Spectra Maxi Germini fluorescence plate reader (Molecular Devices, CA), AMC fluorescence, released by caspase activity, was measured at 460 nm using 360 nm excitation wavelength. Caspase activity was normalized per microgram of protein determined by BCA protein assay kit (Pierce, IL).

Immunoblot analysis

Cell lysates were prepared using SDS lysis buffer (2% SDS, 125mM Tris-HCl, pH 6.8, 20% Glycerol). The lysates were boiled for 5 min and then clarified by a 20-minute centrifugation at 4°C. Protein concentration was measured using BCA protein assay reagent (Pierce, IL). Equal amounts of protein samples in SDS sample buffer (1% SDS, 62.5mM Tris-HCl, pH 6.8, 10% Glycerol, 5% β-mercaptoethanol, 0.05% Bromophenol Blue) were boiled for 5 minutes and subjected to reducing SDS-PAGE. After electrophoresis, the proteins were transferred to a nitrocellulose membrane. The blot was blocked with 5% nonfat dry milk in 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.02% NaN_3 and 0.2% Tween-20 (T-TBS) for 1 hour at room temperature. The membranes were incubated with the appropriate primary antibody in 5% milk in T-TBS. After three washes with T-TBS, the blot was incubated with the appropriate HRP-conjugated secondary antibody. The antigen was detected using the ECL detection system (Pierce, IL) according to the manufacturer's instruction.

Immunofluorescent study

Cells were incubated with 1% BSA for 1 hr, followed by incubation with 0 or 500 ng/ml recombinant TIMP-1 proteins. After washing with PBS 6 times, cells were incubated with rabbit preimmune IgG, or with rabbit anti-TIMP-1 antibody (that recognizes both murine and human TIMP-1 proteins), followed by incubation with anti-rabbit IgG-FITC. After washing with PBS 6 times, cells were fixed and mounted with anti-fade mounting medium containing 1.5 µg/ml DAPI (Vector Laboratory, CA). The cells were examined with a Zeiss LSM microscope in the confocal mode.

Results

(5-1) Downregulation of TIMP-1 expression in MCF10A cells and its effects on apoptosis.

To further study TIMP-1-regulation of human breast epithelial cell survival, we established MCF10A clones in which endogenous TIMP-1 expression is downregulated using an anti-sense construct of TIMP-1 cDNA (AS TIMP-1 MCF10A). Immunoblot analysis of TIMP-1 confirmed significant downregulation of TIMP-1 expression in AS TIMP-1 MCF10A cells as shown in **Fig. 1A&B**. To test whether downregulation of TIMP-1 enhances cell death, we compared the % cell survival of control, AS TIMP-1 MCF10A and TIMP-1 overexpressing MCF10A cells following loss of cell adhesion. As shown in **Fig. 1C**, only ~25% of AS TIMP-1 MCF10A cells remained viable after 18 hr of anoikis, while ~80% of control vector transfected and ~95% of TIMP-1 overexpressing MCF10A cells were viable. After 48 hr, almost none of AS TIMP-1 MCF10A and only ~15% control vector transfected MCF10A cells survived, whereas ~85% of TIMP-1 overexpressing cells remained viable. These results re-confirmed our finding that TIMP-1 levels are critical determinants for apoptosis sensitivity in human breast epithelial cells.

TIMP-1 contains a signal sequence (23-AA long) at the NH_2-terminus, and is secreted through a classical secretory pathway. When tested for the cell survival activity of recombinant TIMP-1 protein in MCF10A cells, only a mild effect was detected. Here, we asked if exogenously-added TIMP-1 has a mild anti-apoptotic activity, because the surface "receptors" in MCF10A cells are pre-occupied with endogenously expressed TIMP-1. To determine the full effects of exogenous TIMP-1, we examined the anti-apoptotic activity of exogenously-added TIMP-1 proteins using AS TIMP-1 MCF10A cells. As shown in **Fig. 1D**, the cell survival effect of exogenously-added TIMP-1 was greatly enhanced in AS TIMP-1 MCF10A cells. We also examined the effect of exogenous TIMP-1 on fibroblast cell survival. We previously observed that bcl-2 overexpression in fibroblast cells does not result in TIMP-1 induction (2), suggesting that TIMP-1 mediated cell survival may be epithelial cell specific, and may not have any effect in fibroblasts. As shown in **Fig. 1D**, recombinant TIMP-1 failed to enhance survival of murine fibroblast cells deficient TIMP-1 expression (TIMP-1-/- fibroblasts, a gift from Dr. Paul Soloway, at Rosewell Park Cancer Institute, Buffalo, NY). These results further support our hypothesis that extracellular TIMP-1, but not TIMP-2, has an anti-apoptotic activity in breast epithelial cells in a cell-type specific manner (but not in fibroblasts).

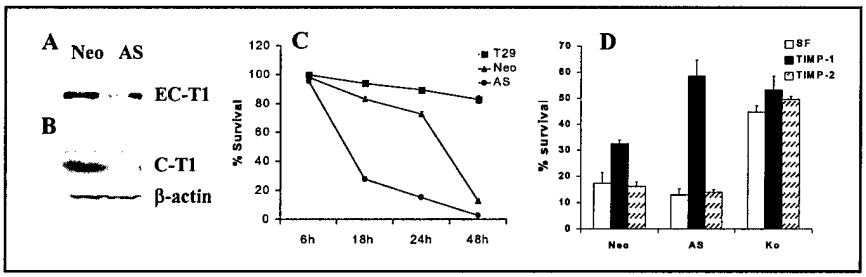

Figure 1. Downregulation of TIMP-1 expression in MCF10A cells and its effects on apoptosis. Conditioned medium (**A**) and cell lysates (**B**) of MCF10Aneo (Neo) and AS TIMP-1 MCF10A (AS) cells were subjected to immunoblot analysis with an anti-TIMP-1 antibody. The bottom panel in B shows the β-actin levels of the same blot reprobed with an anti-human β-actin antibody. **C**; MCF10Aneo (Neo), AS TIMP-1 MCF10A (AS) and TIMP-1 MCF10A #29 (T29) cells were cultured in polyHEMA-coated dishes. At the indicated time points, the number of viable cells was determined by a trypan blue exclusion assay. Cell survival is expressed as a percentage of the respective control cells cultured in regular culture plates. **D**; Apoptosis was induced by culturing MCF10Aneo (neo), AS TIMP-1 MCF10A (AS) and TIMP-1 -/- murine fibroblast (Ko) cells in serum-free medium for 48 hours without (SF), or with 500 ng/ml TIMP-1 or TIMP-2 proteins. The percentage of cell survival was determined by MTT assay, normalized to the MTT signals in the respective cells cultured in serum containing medium (cont). Shown are the means ± S.E. of the triplicate experiments.

(5-2) TIMP-1 regulates caspase activity.

TIMP-1 inhibits cleavage of poly(ADP-ribose) polymerase (PARP) following anoikis induction (2), suggesting that TIMP-1 downregulates caspase activity. Since caspases including caspase-3 and -7 cleave PARP at the $DEVD^{216}$-G site, DEVDase activity was determined by fluorescence released from the tetrapeptide substrate Ac-DEVD-amc. DEVDase activity greatly increased in AS TIMP-1 MCF10A cells at 18 hr after anoikis induction, while MCF10Aneo cells have mild induction of DEVDase activity. At 24, and 48 hr of

anoikis, both MCF10Aneo and AS TIMP-1 MCF10A cells have high levels of DEVDase activity. In contrast, there was no significant induction of DEVDase activity in TIMP-1 overexpressing cells (**Fig. 2A**).

We then examined whether TIMP-1 can inhibit caspase activity induced by staurosporine, an apoptotic agent that rapidly decreases the transmembrane potential of the mitochondria, resulting in activation of the intrinsic caspase cascade (6,7). Downregulation of TIMP-1 expression significantly enhanced staurosporine-induced DEVDase activity in MCF10A cells, and TIMP-1 overexpression effectively prevented DEVDase activity (**Fig. 2B**).

These studies indicate that TIMP-1 regulates classical apoptotic pathways involving caspases following a variety of apoptotic stimuli.

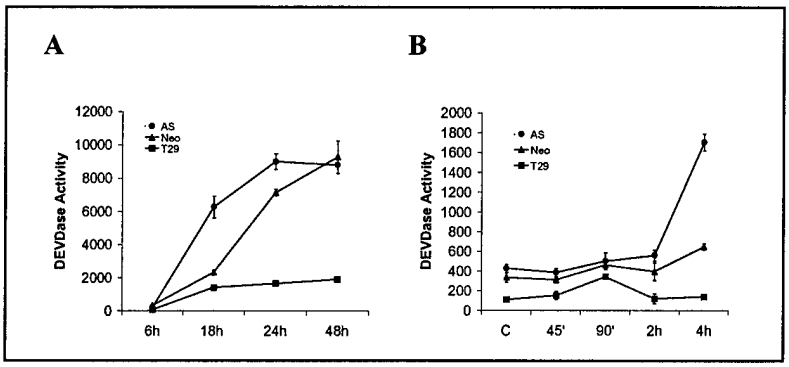

Figure 2. TIMP-1 inhibits DEVDase activity in human breast epithelial cells. Apoptosis was induced in MCF10Aneo (Neo), AS TIMP-1 MCF10A (AS) and TIMP-1 MCF10A #29 (T29) cells by culturing on polyHEMA-coated dishes (**A**), or by treatment with 0.5 μM staurosporine (**B**). At indicated time points, the cells were washed with PBS and lysed with 200 μl lysis buffer as described). After lysates were centrifuged at 16,000 g for 10 min, DEVDase activity in 50 μl cytosol was assayed and the activity was normalized per μg protein. Three independent experiments were performed and the error bars represent standard deviation of the mean of triplicates.

(5-3) Involvement of FAK/PI 3-kinase and MAPK surivial signaling in TIMP-1 inhibition of apoptosis.

Increasing evidence indicates that cell interactions with the ECM transduce biochemical signals mediated, in part, by focal adhesion kinase (FAK) activation (8-11). Constitutively activated forms of FAK (tyrosine phosphorylated form) protect cells against anoikis (12) and free radical-induced cell death (13), suggesting that FAK activity is critical for cell survival. Therefore, we examined whether TIMP-1 anti-apoptotic activity is involved in modulating FAK activity. We found that FAK is more efficiently activated in TIMP-1-overexpressing cells than in the control cells. Interestingly, we also found that FAK is constitutively activated in the cells overexpressing TIMP-1 even in the absence of cell anchorage (2).

FAK is the upstream regulator of the phosphotidylinositol 3-kinase (PI 3-kinase)- serine-threonine kinase/Akt survival pathway (14). Phosphorylated FAK binds to PI 3-kinase and activates its activity, which in turn activates Akt (14,15). We next examined whether TIMP-1 induces cell-survival signal transduction pathways involving FAK/PI 3-kinase/Akt and MAPK activation. As shown in Fig. 3, the levels of active ERKs and PI 3-kinase are indeed significantly higher in TIMP-1 overexpressing MCF10A cells (TIMP-1 MCF10A #29) compared to vector-transfected cells (MCF10Aneo), while TIMP-1 overexpression has little effect on JNKs and p38 (**Figure 3A&C**). More importantly, we also show that exogenously added recombinant TIMP-1 proteins activate ERKs and PI 3-kinase in MCF10A cells (**Figure 3B&D**). *These results further support our working hypothesis that extracellular TIMP-1 induces cell survival pathways involving ERKs and FAK/PI 3-kinase in human breast epithelial cells.*

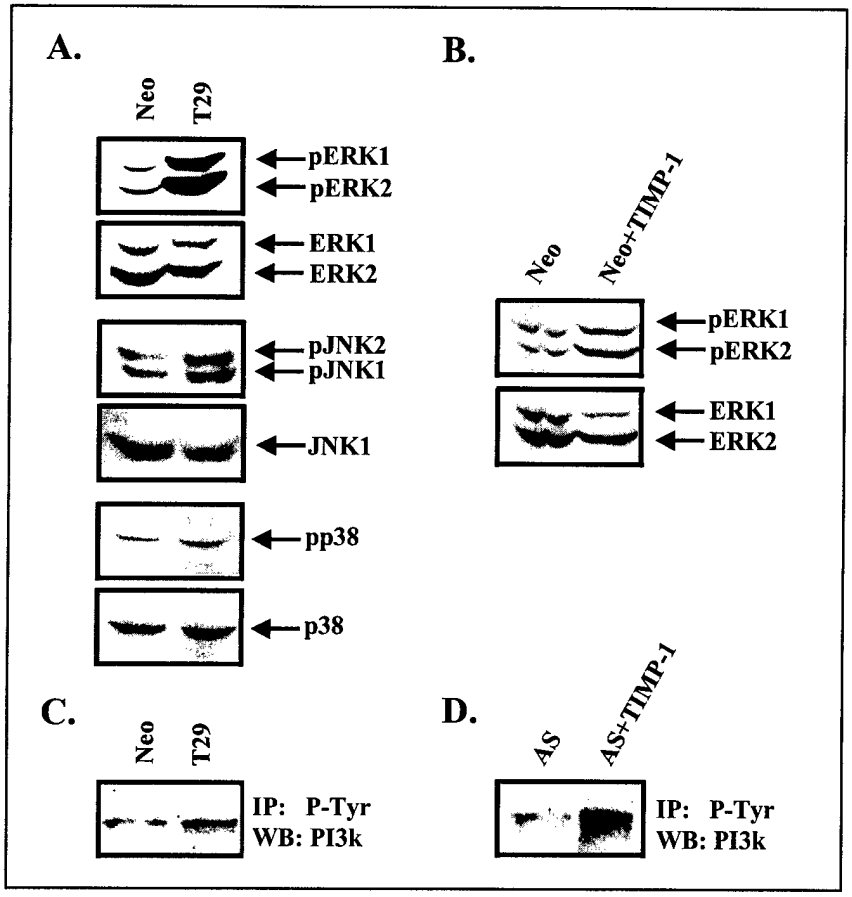

Figure 3. TIMP-1 activates ERKs and PI 3-kinase in human breast epithelial cells. A; Cell lysates (80μg/lane) of 48 hr serum-starved MCF10Aneo (Neo) and TIMP-1 MCF10A #29 (T29) cells were subjected to immunoblot analysis with anti-active ERKs (pERK1/2), anti-ERK1/2, anti-active JNK 1/2 (pJNK1/2), anti-JNK1, anti-active p38 (pp38), anti-p38 antibodies. **B;** MCF10Aneo (Neo) cells were treated without or with 500ng/ml recombinant TIMP-1 proteins for 10 min. Cell lysates (80μg/lane) were subjected to immunoblot analysis with an anti-active ERKs (pERK1/2), anti-ERK1/2 antibodies. **C;** Cell lysates (500μg/lane) of 48 hr serum-starved MCF10Aneo (Neo) and TIMP-1 MCF10A #29 (T29) cells were immunoprecipitated with an anti-phosphotyrosine mAb and protein G agarose beads. The immunopreciptitates were subjected to immunoblot analysis with an anti-PI 3-Kinase p85α polyclonal antibody. **D;** Cell lysates (500μg/lane) of AS TIMP-1 MCF10A (AS) cells without or with 500ng/ml recombinant TIMP-1 treatment for 10 min were immunoprecipitated with an anti-phosphotyrosine mAb and subjected to immunoblot analysis with anti-PI 3-Kinase p85α polyclonal antibody.

(5-4) PI 3-kinase and MAPK survival signaling pathways are critical for TIMP-1 inhibition of cell death. To investigate the role of PI 3-kinase for TIMP-1 mediated cell survival, we examined TIMP-1-mediated cell survival in the presence and absence of wortmannin, an inhibitor of PI 3-kinase (Calbiochem, CA). As shown in **Fig. 4A**, TIMP-1 mediated cell survival was almost completely abolished by wortmannin in TIMP-1 overexpressing MCF10A cells. Similarly, exogenously added TIMP-1-mediated cell survival in AS TIMP-1 MCF10A cells was abolished by the wortmannin treatment (**Fig. 4B**). Since studies suggest significance of cross-talk between the dual signaling pathways, PI 3-kinase-Akt and MEK1-MAPK pathways, for the regulation of cell survival/death (16,17), we also examined the effect of PD98059 (an inhibitor of MEK1, Calbiochem) on TIMP-1 inhibition of cell death. Inhibition of MEK1/MAPK pathway reduced TIMP-1 overexpressing cell survival following growth-factor withdrawal (**Fig. 4A**). Similarly, PD98059 treatment significantly reduced exogenous TIMP-1-mediated cell survival in AS MCF10A cells, while PD98059 or wortmannin treatment alone had no cytotoxicity in these cells (**Fig. 4B**).

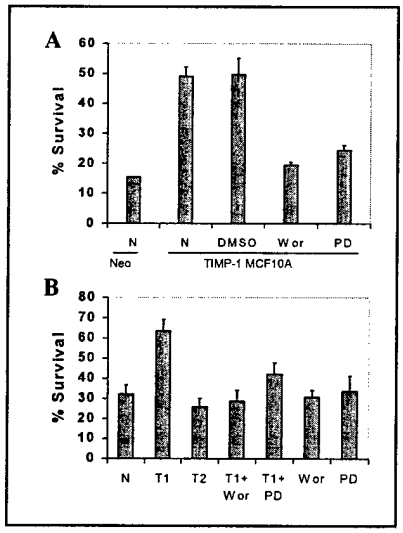

Figure 4. PI 3-kinase and MAPK are critical for TIMP-1 mediated cell survival. A; The control vector transfected (Neo) and TIMP-1 overexpressing MCF10A cells (TIMP-1 MCF10A) were cultured in serum-free medium without (N), or with vehicle only (DMSO), 200 nM wortmannin (wor) or 10 μM PD98059 (PD) for 48 hours. The percentage of cell survival was determined by MTT assay, normalized to the MTT signals in the respective cells cultured in serum containing medium. Shown are the means ± S.E. of the triplicate experiments. **B;** AS TIMP-1 MCF10A cells were cultured in serum free medium in the absence (N) or presence of 500 ng/ml recombinant TIMP-1 (T1), 500 ng/ml recombinant TIMP-2 (T2), TIMP-1 and 200 nM Wortmannin (T1+Wor), TIMP-1 and 10 μM PD98059

(T1+PD), 200 nM Wortmannin (Wor), or 10 μM PD98059 (PD). After 48 hr, the percentage of cell survival was determined as described above.

These results suggest that PI 3-kinase and MAPK signaling pathways are critical for transfected TIMP-1- or exogenously added TIMP-1-mediated human breast epithelial cell survival.

(5-5) TIMP-1 binds on the MCF10A cell surface.

The anti-apoptotic effect of the exogenous TIMP-1 suggested that TIMP-1 binding to the cell surface activates cell survival signaling in human breast epithelial cells. To examine whether TIMP-1 localizes on the cell surface, we used immunofluorescence analysis using confocal microscopy. To this end, non-permeabilized MCF10A cells, which express endogenous TIMP-1, were immunostained with a specific rabbit pAb to TIMP-1. As shown in **Fig. 5C**, MCF10A cells exhibit a punctate pattern of TIMP-1 on the cell periphery indicating that TIMP-1 is associated with the cell surface. Addition of exogenous recombinant TIMP-1 to the MCF10A cells resulted in an enhanced signal consistent with binding of TIMP-1 protein to the cell surface (**Fig. 5D**). These stainings were specific since without primary antibody (**Fig. 5A**) or use of preimmune rabbit IgG (**Fig. 5B**) resulted in lack of signals. Taken together, these studies demonstrate that the endogenous TIMP-1 is present on the surface of MCF10A cells and that addition of recombinant TIMP-1 results in association of TIMP-1 with the cell surface. In a control experiment, TIMP-1 was not detected in TIMP-1 -/- murine fibroblasts, as expected (**Fig. 5E**). Consistent with the result shown in **Fig. 5D** (TIMP-1 -/- fibroblasts do not respond to the anti-apoptotic activity of TIMP-1), exogenously added recombinant TIMP-1 proteins failed to associate with cell surface components in TIMP-1 -/- fibroblasts (**Fig. 5F**).

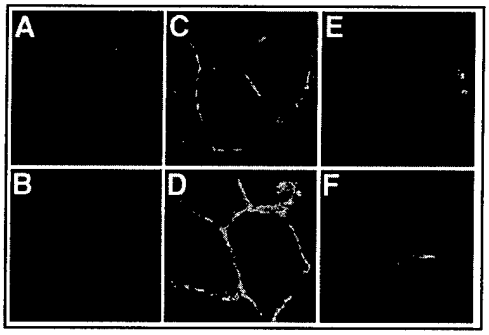

Figure 5. Immunofluorescence study of the TIMP-1 protein on MCF10A cell surface. MCF10A (**A-D**) and TIMP-1 -/- murine fibroblast (**E&F**) cells were incubated with 1% BSA for 1 hr, followed by incubation with 0 (**A,B,C,E**) or 500 ng/ml recombinant TIMP-1 proteins (**D&F**). After washing with PBS for 6 times, cells were incubated without (**A**), with rabbit preimmune IgG (**B**), or with rabbit anti-TIMP-1 antibody (that recognizes both murine and human TIMP-1 proteins) (**C-F**), followed by incubation with anti-rabbit IgG-FITC (**A-F**). After washing with PBS for 6 times, cells were fixed and mounted with anti-fade mounting medium containing 1.5 μg/ml DAPI (Vector Laboratory, CA). The cells were examined with a Zeiss LSM microscope in the confocal mode. The nuclei stained with DAPI were shown in blue, and TIMP-1 proteins in green.

(5-6) TIMP-1 specifically binds to a 150-kDa surface protein in MCF10A cells.

To identify TIMP-1 binding protein(s) on the surface of MCF10A cells, the cells were surface biotinylated with sulfo-NHS-biotin (Pierce, IL). The lysates were subjected to immunoprecipitation with the pAb to TIMP-1 and the immunoprecipitates were resolved by SDS-PAGE followed by detection of the biotinylated proteins with avidin-HRP. **Fig. 6A** (lane 4) shows that the pAb to TIMP-1 specifically precipitated biotinylated TIMP-1 (~29 kDa), as expected, and an additional biotinylated protein of ~150-kDa (arrow). These proteins were not detected in non-biotinlylated cells (**Fig. 6A**, lane 2) demonstrating that the signal was not due to non-specific binding of the avidin-HRP. Furthermore, preimmune IgG failed to immunoprecipitate these proteins (**Fig. 6A**, lane 3) confirming the specificity of the signals. The ~60 kDa protein (marked with an aterisk) represents a non-specific band as it was also detected with the preimmune IgG. To confirm that the biotinylated ~29 kDa protein (T1 in **Fig. 6A**, lane 4) was indeed TIMP-1, and to ensure that the co-immunoprecipitation efficiencies between biotinylated and non-biotinylated lysates were comparable, the same blots were reprobed with the anti-TIMP-1 antibody. This experiment confirmed that the ~29 kDa protein (T1, lane 4) as TIMP-1 protein, and that the efficiencies of the immunoprecipitations were comparable (**Fig. 6B**).

Ligand blot analyses were also used to identify TIMP-1 binding proteins (**Fig 6C**). Briefly, cell lysates of MCF10A cells were subjected to SDS-PAGE analysis and transferred to a nitrocellulose membrane. The filter strips were then incubated without (**Fig. 6C**, lane 1) or with ~500 ng/ml biotinylated recombinant TIMP-1

protein (**Fig. 6C**, lane 2). Proteins interacting with biotinylated TIMP-1 were visualized by avidin-HRP detection. These studies identified a protein of ~150-kDa that was specifically detected in the presence of TIMP-1. The ~70- kDa band (§) represents a non-specific protein since it was observed in the absence of TIMP-1.

These data demonstrate that TIMP-1 can be detected on the surface of MCF10A cells and that a ~150-kDa surface protein (refereed to as p150) forms a specific complex with TIMP-1.

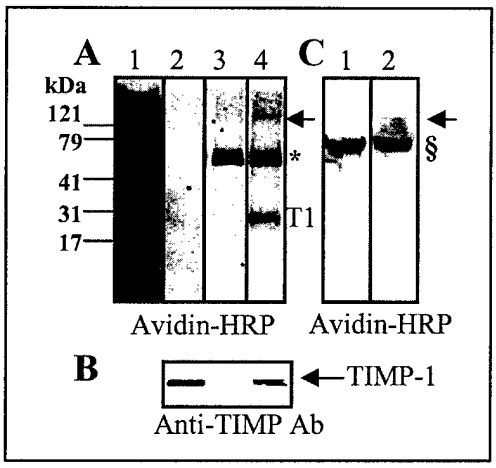

Figure 6. Identification of the putative TIMP-1 binding protein on the cell surface. **A**; Ten μg of surface-biotinylated MCF10A lysates were subjected to SDS-PAGE analysis (lane 1). Five hundred μg of non-biotinylated (lane 2) and cell-surface biotinylated (lane 3&4) MCF10A cell lysates were subjected to a co-immunoprecipitation experiment with anti-TIMP-1 antibody (lane 2&4) or with rabbit preimmune IgG (lane 3). TIMP-1 complexes were detected by streptavdin-HRP. **B;** The same filter of panel A was subjected to immunoblot analysis using anti-TIMP-1 antibody. **C**; A ligand blot analysis: Fifty μg of total cell lysates were subjected to SDS-PAGE analysis, transferred to a nitrocellulose membrane, and the strips of lane 1 and lane 2 were incubated with 5% non-fat milk in TTBS, followed by incubation with 0 (lane 1) and 500 ng/ml biotinylated TIMP-1 proteins (lane 2). TIMP-1 binding proteins were detected by avidin-HRP.

(5-7) Analysis of TIMP-1 domain critical for apoptosis regulation.

To further determine whether endogenous TIMP-1 has anti-apoptotic activity or TIMP-1 secretion is required for apoptosis inhibition, we generated two constructs encoding full-length TIMP-1/FLAG fusion proteins with and without the 23AA-long signal sequence necessary for secretion. These TIMP-1/FLAG constructs were introduced into human embryonic kidney 293 cells that express endogenous TIMP-1 proteins at barely detectable levels. As shown in **Figure 7**, TIMP-1/FLAG fusion proteins containing the leader sequence were detected both in cell lysates (intracellular TIMP-1) and in conditioned medium (extracellular TIMP-1). Of note, two forms of TIMP-1 proteins were detected as previously reported (2), although the nature of these forms are unclear at present. In contrast, secretion deficient (SD) TIMP-1/FLAG fusion protein lacking 23 AA leader sequence was detected only in cell lysates, but not in conditioned medium. *These results show that deletion of the 23 AA leader sequences effectively prevents TIMP-1 secretion.* We are currently investigating whether intracellular TIMP-1 has anti-apoptotic activity in 293 cells. We will also examine anti-apoptotic activity of intracellular and extracellular TIMP-1 proteins in human breast epithelial cells

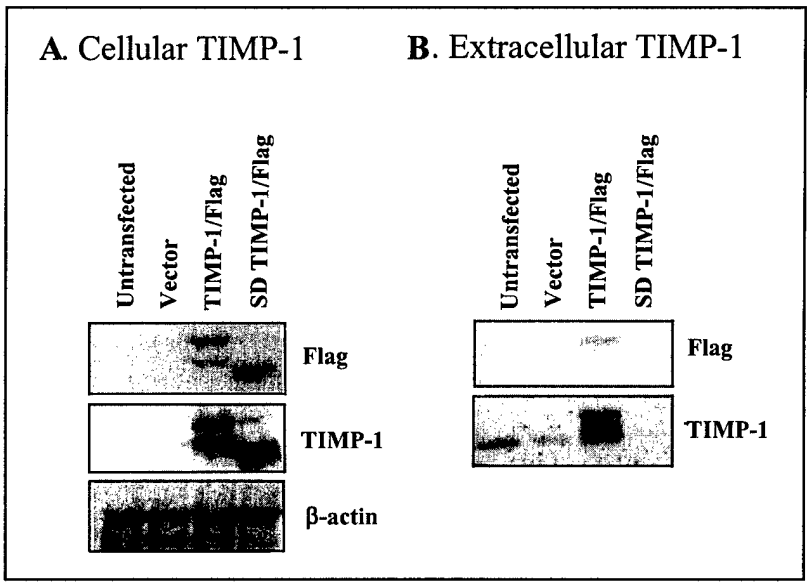

Figure 7. Deletion of the N-terminal 23 AA signal sequence prevents TIMP-1 secretion. Human embryonic kidney 293 cells were transfected with p3XFLAG-CMV-14 vector containing no cDNA insert (Vector), cDNA insert encoding full-length TIMP-1/FLAG fusion proteins with (TIMP-1/Flag) or without the 23AA signal sequence for secretion (SD TIMP-1/Flag) by the calcium phosphate method. Cell lysates (**A**) and conditioned medium (**B**) were collected from untransfected and transfected cells, and subjected to immunoblot analysis with anti-FLAG M2 mAb and anti-TIMP-1 polyclonal antibody. The bottom panel in A shows the β-actin levels of the same blot reprobed with an anti-human β-actin antibody.

(6) Key Research Accomplishments

The progress report presented here demonstrates that (i) endogenously expressed TIMP-1 or exogenous TIMP-1 can protect against apoptosis induced by a variety of apoptotic insults; (ii) donwregulation of TIMP-1 expression enhances apoptosis in human breast epithelial cells; (iii) TIMP-1 constitutively activates focal adhesion kinase (FAK)/PI 3-kinase and MAPK signaling pathways; (iv) both FAK/PI 3-kinase and MAPK signaling pathways are critical for TIMP-1 mediated cell survival; (v) TIMP-1 binds to the cell surface of MCF10A cells; and (vi) a 150-kDa surface protein binds TIMP-1. Based on these results, our current working hypothesis is that TIMP-1 binding on the cell surface, induces a signal transduction pathway leading to FAK and MAPK activation. Active FAK induces PI 3-kinase/Akt and MAPK survival pathway, which regulates the apoptosis commitment step, thereby effectively inhibits apoptosis induced by a variety of apoptotic stimuli.

(7) Reportable Outcomes

Publications:

Liu, X-W., Fridman, R. and Kim, H.-R. C. Extracellular TIMP-1 activates FAK/PI 3-kinase and MAPK signal transduction pathways critical for apoptosis regulation in human breast epithelial cells. Manuscript in preparation.

(8) Conclusion

Taken together, our study suggests a new paradigm in the regulation of apoptosis in human breast epithelial cells, which is mediated by TIMP-1. Our study will contribute to the collective endeavor to understand the molecular mechanisms regulating apoptosis and the role of TIMP-1 during development and pathogenesis, and to develop novel approaches for controlling apoptosis regulating gene functions in breast cancer

(9) References

1. Upadhyay, S., Li, G., Liu, H., Chen, Y. Q., Sarkar, F. H., and Kim, H. R. (1995) *Cancer Res* **55**(20), 4520-4
2. Li, G., Fridman, R., and Kim, H. R. (1999) *Cancer Res* **59**(24), 6267-75
3. Yoshiji, H., Gomez, D. E., and Thorgeirsson, U. P. (1996) *Int J Cancer* **69**(2), 131-4
4. McCarthy, K., Maguire, T., McGreal, G., McDermott, E., O'Higgins, N., and Duffy, M. J. (1999) *Int J Cancer* **84**(1), 44-8
5. Ree, A. H., Florenes, V. A., Berg, J. P., Maelandsmo, G. M., Nesland, J. M., and Fodstad, O. (1997) *Clin Cancer Res* **3**(9), 1623-8
6. Nomura, K., Imai, H., Koumura, T., Arai, M., and Nakagawa, Y. (1999) *J Biol Chem* **274**(41), 29294-302
7. Samali, A., Cai, J., Zhivotovsky, B., Jones, D. P., and Orrenius, S. (1999) *Embo J* **18**(8), 2040-8
8. Ruoslahti, E., and Reed, J. C. (1994) *Cell* **77**(4), 477-8
9. Hynes, R. O. (1992) *Cell* **69**(1), 11-25
10. Guan, J. L., and Shalloway, D. (1992) *Nature* **358**(6388), 690-2
11. Lipfert, L., Haimovich, B., Schaller, M. D., Cobb, B. S., Parsons, J. T., and Brugge, J. S. (1992) *J Cell Biol* **119**(4), 905-12
12. Frisch, S. M., Vuori, K., Kelaita, D., and Sicks, S. (1996) *J Cell Biol* **135**(5), 1377-82
13. Sonoda, Y., Kasahara, T., Yokota-Aizu, E., Ueno, M., and Watanabe, S. (1997) *Biochem Biophys Res Commun* **241**(3), 769-74
14. Sonoda, Y., Watanabe, S., Matsumoto, Y., Aizu-Yokota, E., and Kasahara, T. (1999) *J Biol Chem* **274**(15), 10566-70
15. Chen, H. C., Appeddu, P. A., Isoda, H., and Guan, J. L. (1996) *J Biol Chem* **271**(42), 26329-34
16. Almeida, E. A., Ilic, D., Han, Q., Hauck, C. R., Jin, F., Kawakatsu, H., Schlaepfer, D. D., and Damsky, C. H. (2000) *J Cell Biol* **149**(3), 741-54.

17. De Nichilo, M. O., Katz, B. Z., O'Connell, B., and Yamada, K. M. (1999) *J Cell Physiol* **178**(2), 164-72.